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TITLE: Development of a Novel Vector for Multiple CDC Category a Pathogens

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14. ABSTRACT

Specific Aim 1 was to generate a panel of RhCMV/MPV vectors expressing MPV antigens A29L, A35R, M1R and B6R in either the wild type RhCMV vector, or in a vector lacking MHC immunomodulatory genes. Vectors have been constructed and characterized, and we have subsequently selected one WT RhCMV vector (WTRhCMV/A35R) for immunogical characterization in rhesus macaques. Specific Aims 2 and 3 were to establish the pathobiology of WT MPV infection in RMs, and to monitor the immunological consequences of WT MPV infection. To date, six RMs have been experimentally inoculated intrabronchially with MPV Zaire strain. Two with 2 x 107 plaque forming units (PFU) and four with 2 x 105 PFU, to define a lethal dose by this route of infection and to characterize the virus/host interactions.

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ABSTRACT:
The long-term goal of this project is to develop and evaluate human cytomegalovirus (HCMV) as an effective large capacity persistent vaccine vector to provide protective immunity for multiple bioterrorist agents and emerging diseases. The aim of the current proposal was to determine the potential utility of HCMV as vaccine vector for CDC category A agents using rhesus cytomegalovirus (RhCMV) vaccine vectors in combination with the monkeypox (MPV)-rhesus macaque (RM) model. RhCMV is highly homologous to HCMV, and the MPV:RM model recapitulates all aspects of smallpox infection of humans. **Specific Aim 1** was to generate a panel of RhCMV/MPV vectors expressing MPV antigens A29L, A35R, M1R and B6R in either the wild type RhCMV vector, or in a vector lacking MHC immunomodulatory genes. Vectors have been constructed and characterized, and we have subsequently selected one WT RhCMV vector (WTRhCMV/A35R) for immunogical characterization in rhesus macaques. **Specific Aims 2 and 3** were to establish the pathobiology of WT MPV infection in RMs, and to monitor the immunological consequences of WT MPV infection. To date, six RMs have been experimentally inoculated intrabronchially with MPV Zaire strain. Two with 2×10^7 plaque forming units (PFU) and four with 2×10^5 PFU, to define a lethal dose by this route of infection and to characterize the virus/host interactions. A summation of the ongoing studies is provided. Together, completion of these three specific aims will form the foundation for future studies designed to determine the efficacy of the RhCMV/MPV vectors at inducing a protective immune response to MPV challenge in RMs.

INTRODUCTION:
The aim of the current study is to ascertain the potential utility of human cytomegalovirus (HCMV) as a safe, potent, large capacity vaccine vector for CDC category A agents. HCMV possesses a number of unique characteristics, which potentially make HCMV an ideal vaccine vector to induce protective immunity against acute and chronic bioterrorist weapons. These characteristics include the lack of significant disease associated with infection, persistence of the virus for the lifespan of the host, an ability to re-infect HCMV seropositive individuals, and a capacity to induce a large memory T cell response. In the present study, we will determine the utility of the CMV vector approach using the highly homologous rhesus cytomegalovirus (RhCMV) vaccine vector in combination with the monkeypox (MPV)-rhesus macaque (RM) model. The MPV:RM model is the only animal model that recapitulates all aspects of smallpox virus infection in humans. Since MPV also infects humans and causes symptoms that are indistinguishable from smallpox, MPV was also recently added to the list of category A select agents. Vaccinia virus (VV) is an effective vaccine against small pox virus and MPV, but has significant side effects in a portion of the human population. Modified vaccinia ankara (MVA) vaccination has recently been shown to be partially protective against MPV in non-human primates, but required two vaccinations in order to induce protection. In the event of an actual smallpox outbreak, this approach would be unfeasible since there would be no time for multiple vaccinations to be made in close contacts and the effectiveness of this highly attenuated vaccine for post-exposure vaccination is uncertain at best. Therefore, development of long-term protection against smallpox virus and MPV in humans with a safe vaccine that can be administered in a single dose would be of significant value. Recently, subunit DNA vaccine vectors expressing multiple VV genes (A27L, A33R, L1R, and B5R) were shown to protect mice and non-human primates from lethal challenge with MPV(2, 3). Specific Aim 1 of the current study is to generate RhCMV vectors expressing MPV correlates of A27L, A33R, L1R, B5R (A29L, A35R, M1R and B6R) in both a wild type RhCMV vector as well as in a vector deleted for immunoregulatory regions. Specific Aim 2 and 3 are designed to further characterize the virological and immunological aspects of MPV infection of RM to further develop the MPV:RM model.

BODY:

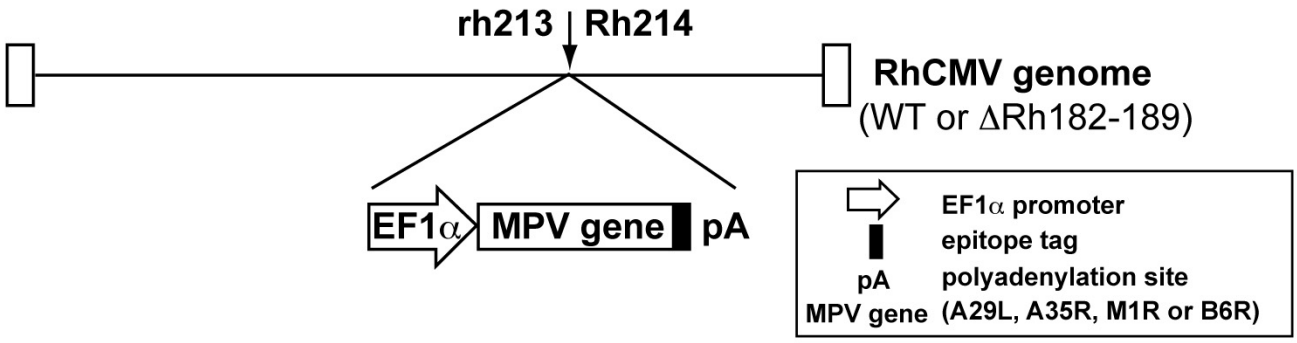
Specific Aim 1: We will generate RhCMV-MPV vectors expressing the VV protective antigens that correlate to MPV-A29L, A35R, M1R and B6R.

SA1A: *We will first construct a wild type (WT) RhCMV vector expressing the MPV protective antigens.*

SA 1B: *We will develop an RhCMV vector that lack the MHC immunomodulatory genes expressing the MPV protective antigens to determine if we can increase immunogenicity as well as increase vector space for other antigen expression cassettes."*

Specific Aim 1 has been completed resulting in construction of two series of RhCMV vectors each containing one of four individual MPV genes (A29L, A35R, M1R and B6R). These MPV genes are the correlates of VV genes that were shown to induce a protective immune response to MPV in RMs using a DNA vaccine approach (3). Two different RhCMV genetic backgrounds were used for construction of these vectors comprised of either the complete WT genome (designated WTRhCMV/MPV vectors), or a genome deleted for major immunomodulators of the virus (US2-US11 homologous region Rh182-189) (designated ΔRhCMV/MPV vectors). A schematic of the RhCMV/MPV vectors is shown in **Figure 1**.

Figure 1. Schematic of RhCMV/MPV vectors.



The MPV gene cassettes were inserted into RhCMV vectors using bacterial artificial chromosome (BAC)-based linear recombination technology. Briefly, recombination cassettes comprised of the EF1α expressed epitope-tagged MPV genes combined with a selectable marker (kanamycin resistance; Kan^R) were inserted at the desired site within the RhCMV BAC genome using linear recombination. Recombinant RhCMV BAC clones were then selected on the basis of kanamycin resistance. The Kan^R marker is flanked by *frt* recombination sequences, which enables removal of the Kan^R by FLP recombinase leaving only the MPV gene and a single *frt* site within the recombinant RhCMV BAC genome. During the last year we have finished construction and genomic characterization of ΔRhCMV/MPV and WTRhCMV/MPV series vectors. Restriction enzyme digestion analysis shows the lack of aberrant genomic rearrangements in the recombinant RhCMV BAC vectors (compare EtBR stained gel of RhCMV vectors with wild type RhCMV) (**Figure 2A**).

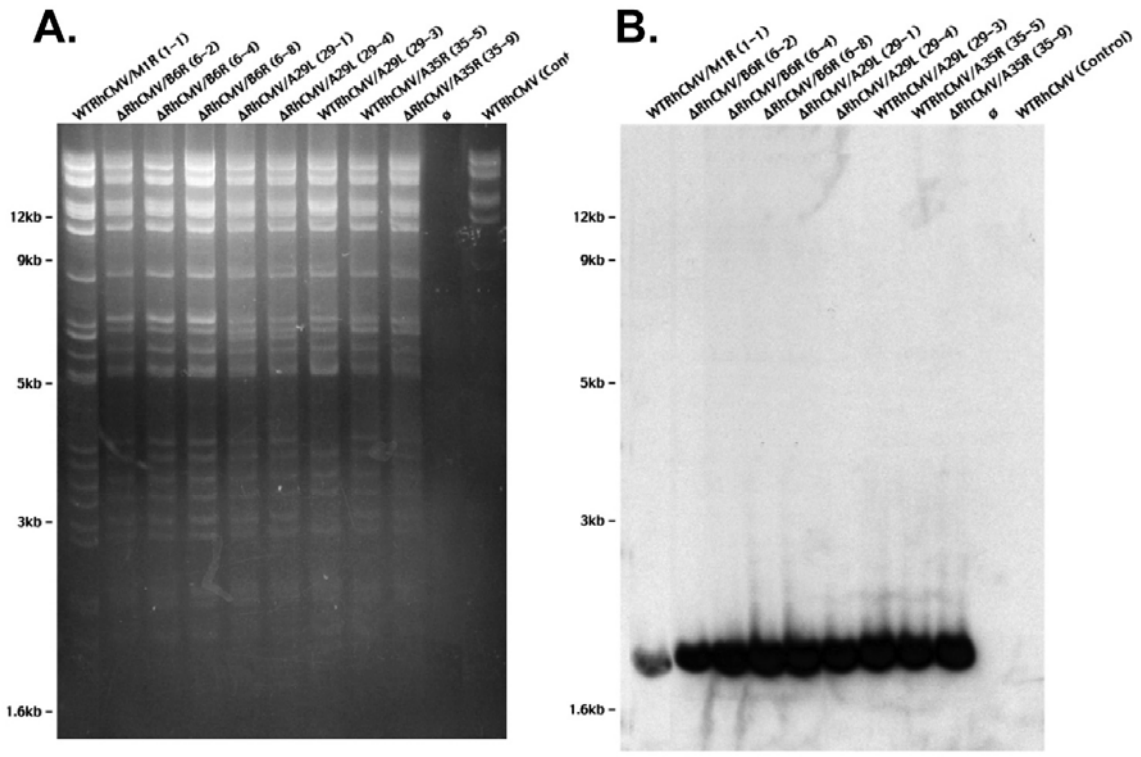


Figure 2: Genomic characterization of RhCMV/MPV BAC vectors. Linear recombination was used to insert a cassette, composed of an EF1α promoter-driven MPV gene and a *frt*-flanked Kan^R marker, into the RhCMV BAC (either WTRhCMV or ΔRhCMV). The Kan^R marker was subsequently removed by FLP recombinase. A) Restriction enzyme digestion analysis shows the lack of aberrant genomic rearrangements in the recombinant RhCMV BAC vectors. B) Southern analysis using a probe directed against the Kan^R shows insertion of the recombination cassette within the RhCMV BAC genome.

Southern analysis using a probe directed against the Kan^R shows insertion of the recombination cassette within the RhCMV BAC genomes (**Figure 2B**). Presumably due to size limitations on the process of linear recombination, we were unable to obtain the construct containing the largest of the MPV genes, B6R, in the WTRhCMV background. The Kan^R marker was subsequently removed from all BAC clones by FLP induction, and virus was reconstituted by transfection of BAC DNA into RhCMV-permissive RM fibroblasts.

Although beyond the scope of the initial proposal, we are proceeding to perform preliminary studies to determine the capacity of RhCMV/MPV vectors to induce an immune response against the MPV target antigens in RMs. For these studies, we have selected the WTRhCMV/MPV vector expressing A35R (WTRhCMV/A35R) for further immunological characterization. A western of lysates from rhesus macaque fibroblasts infected with WTRhCMV/A35R shows high levels of A35R protein expression (**Figure 3**). Although the ΔRhCMV/A35R showed a similar high level of protein expression (Figure 3), recent results from our ongoing studies using the SIV model system indicate that deletion of immune modulators may severely compromise the ability of the virus to reinfect the RhCMV seropositive host (Dr. K Fruh, personal communication). Therefore, the WT RhCMV vector background was selected for these immunological analyses. Together, these experiments represent an exciting conclusion to this proposal and will determine the potential efficacy of the RhCMV vector approach for inducing protective immunity to pox viruses.

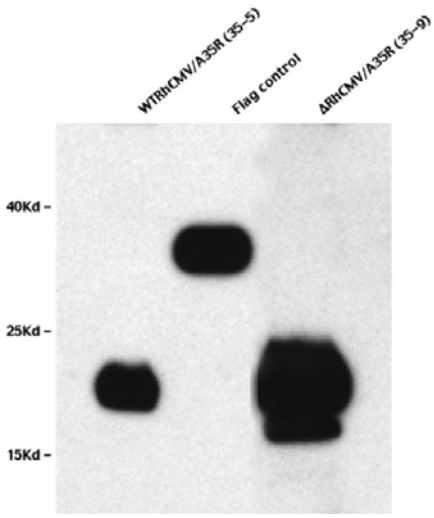


Figure 3. Western analysis of MPV gene expression from RhCMV/A35R viruses. Viruses (both Δ and WT versions) were reconstituted by transfection of BAC DNA into RhCMV-permissive RM fibroblasts. Cells were harvested when reconstituted virus had spread through the culture, as observed by extensive virus-associated cytopathic effect. Expression of A35R protein was determined using antibodies directed against the epitope tag. An epitope tag-reactive band of the size predicted for the tagged A35R protein confirms expression of the MPV protein in RhCMV/A35R viruses.

Specific Aim 2: We will establish the pathobiology of WT MPV infection of nonhuman primates

A. Experimental inoculation and clinical evaluation

During the second year of support we further evaluated our intrabronchial infection model for monkeypox virus pathogenesis in RMs. For these studies, we inoculated two RM with 2×10^5 plaque forming units (PFU) of MPV Zaire (MPVZ), which earlier resulted in the death of one of two RM. This dose was chosen to confirm if this is a lethal dose fifty (LD_{50}) by this route of infection and to provide more clinical data over an entire infection period (exposure, acute infection, clinical disease and convalescence).

To accurately measure febrile responses to MPV infection, both animals were implanted with Mini-Mitter series 3000 transmitters, which measure body temperature and animal activity and send the information to a receiver connected to a computer. The transmitters were implanted seven days before experimental MPV infection to allow the animals to heal from a lymph node biopsy and to provide baseline readings for each animal prior to infection as controls. Normal sedation and rectal temperature measurement is not an accurate measurement as animals are easily agitated, yielding higher body temperatures. Thus, biotelemetry provides a more reliable method to measure temperature throughout the study period 24 hours a day.

Utilizing the biotelemetry system, we were able to monitor increases in body temperature for both animals. On day 5 post-infection, body temperature for both animals increased by 1^oC and peak temperatures were detected on day 12 post-infection for animal 23358 (38.6^oC) and day 18 post-infection for animal 23218 (40.2^oC) **(Figure 4)**. Animal 23218 exhibited two febrile cycles; cycle 1, day 5 pi to day 15 pi, and cycle 2, day 16 pi to day 21 pi. Body temperatures returned to near pre-infection levels by day 21 post-infection. Actual core body temperatures were higher than those obtained with the transmitter, as the transmitters are place directly under the skin, which is $2\text{-}3^oC$ lower than core temperature.

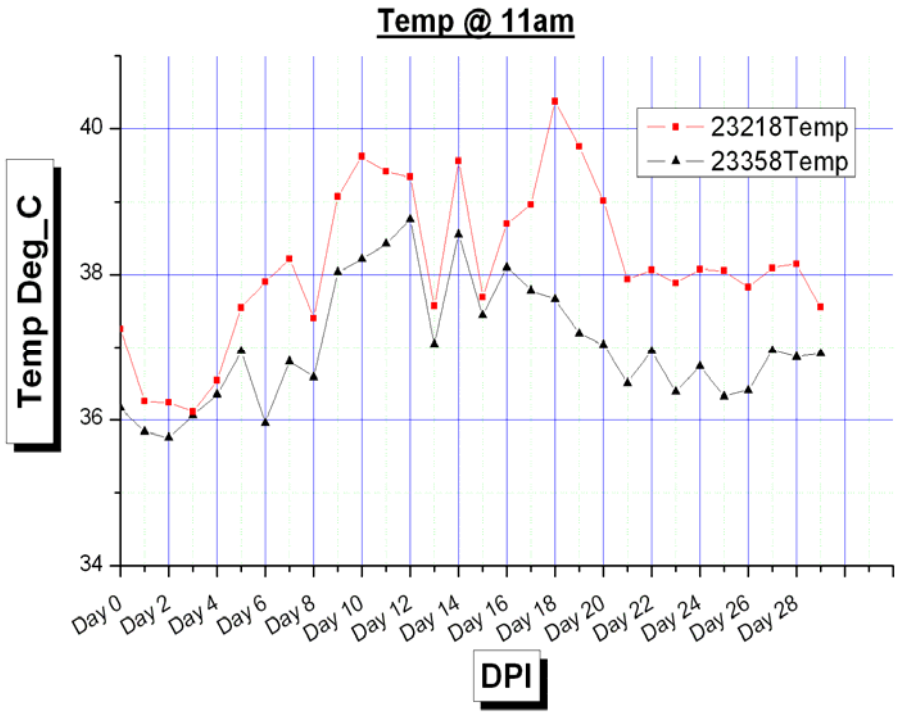


Figure 4 Temperature of each animal at 11:00 AM each day utilizing biotelemetry equipment. Animal 23218's temperature is shown in red and animal 23358's temperature is in black.

As with the previous animal infections, both animals exhibited episodes of coughing and both displayed oral pox lesions. By day 7 pi, the animals exhibited numerous lesions in and around their mouths and both had persistent cough and symptoms of pneumonia by clinical examination. On day 11 pi, the lesions on both animals were progressing, and their oxygen saturation levels decreased to 90%. By day 12 pi, the oxygen saturation levels dropped to 85%. During days 13 through 14, animal 23358's condition stabilized, whereas animal 23218's condition worsened, most likely due to secondary bacterial infection. Treatment with broad spectrum antibiotics resolved the infection in 23218.

The two animals also developed monocytosis, as has been described by Zaucha et al., (5). Specifically, the both animals showed mark increase (near 3-fold) in circulating monocytes by day 7 pi and peak levels by day 12pi (near 9-fold), which returned to pre-infection levels by day 25 pi (**Figure 5**). The increase in monocytes was further confirmed by fluorescent activated cell sorter (FACS) analysis using CD14 as a marker for monocytes.

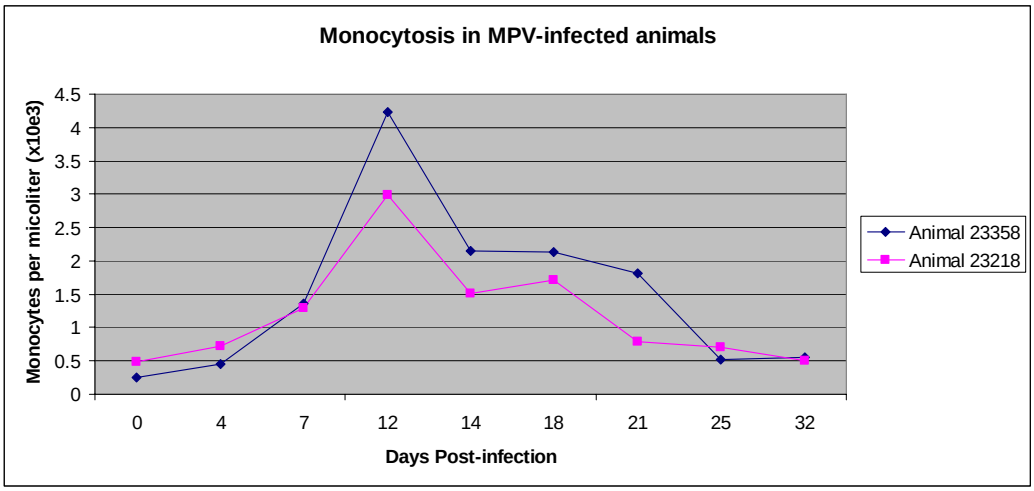


Figure 5 Monocytosis in MPV-infected animals.

B. Gross and histopathological examination

Both animals were subsequently euthanized on day 32 pi and showed little evidence of MPV lesions on their extremities or internal organs as was observed in our earlier cohorts.

C. Virus isolation

Peripheral blood mononuclear cells (PBMCs) yielded recoverable virus by co-culture in the two animals. Interestingly viral loads coincided with the increase in circulating monocytes.

We were also able to recover replicating MPV from the fluids recovered by bronchoalveolar lavage (BAL). Further evaluation of viral load in BAL and blood is being performed by real-time PCR.

Specific Aim 3: We will monitor the immunological consequences of WT MPV infection of non-human primates

SA3A*: We will determine the antiviral CD4+ and CD8+ T cell responses to the MPV structural proteins, A29L and A35R, in MPV-infected RM.*

SA3B: *We will determine the antiviral antibody response mounted against the MPV structural proteins, A29L and A35R, in MPV-infected RM.*

Utilizing immunological reagents that are well characterized for RMs (4) we initiated immunological analysis on the two MPV-infected RM. We first confirmed the kinetic response of the immune system to infection by Ki-67 staining. The data were consistent with that of the second cohort. Specifically, CD4+ central memory T cells increased on day 7 pi, which was followed by an increase on day 11 of CD4+ effector memory T cells, CD8beta+ T cell of the effector memory type and both marginal zone and memory CD20+ B cells.

We subsequently analyzed antigen specific T cell responses following MPV challenge as described by Hammarlund et al., (1). Briefly, peripheral blood mono-nuclear cells (PBMC) or bronchial alveolar lavage (BAL) cells from either infected or naïve animals were stimulated with either vaccinia (WR strain) or monkey pox virus at an MOI of 1. After a 12 hr incubation, brefeldin A was added to block cytokine export from the endoplasmic reticulum /Golgi for an additional 6hr. Cells were then washed and stained with anti CD8, CD4, CD95 and CD28 to delineate T cell subsets. Both CD8 and CD4 T cells can be further subdivided into three subsets based on the pattern of expression of CD28 and CD95: 1) naïve (CD28+CD95-); 2) central memory (CD28+CD95+); and 3) effector memory (CD28-CD95+). After the surface staining, the cells were permeabilized and antibodies against IFNγ and TNF-α were added. All samples were acquired on the LSRII using the FACsDIVA software (Becton Dickenson, San Jose, CA) and analyzed using FlowJo (Treestar, Ashland, OR).

Our data shows that following MPV infection animals generate a strong T cell response dominated by the effector memory (EM) CD8 subset (**Fig6A**). Interestingly, EM CD8 T cells present in PBMC secrete higher levels of IFNγ in response to vaccinia stimulation than MPV stimulation. This might be indicative of immune evasion mechanisms by MPV that dampen the adaptive immune response in the periphery. This difference, however was not observed in the CD8 T cells obtained from the BAL (**Fig6B**) where the IFNγ responses generated after MPV or VV infection were equivalent (**Fig7A**). This discrepancy could be due to the different antigen presenting cell populations present in the lung versus peripheral blood. Alternatively this difference could be due to the difference in activation status of the T cells recruited to the site of infection (lung) versus those that circulate in peripheral blood. We are currently investigating these different possibilities by studying the levels of both MHC class I and II molecules following VV and MPV infection in PBMC and BAL cells. We are also analyzing the resident cell populations in the lung that could act as antigen-presenting cells using multicolor flow cytometry.

Peripheral CD4 T cells generated a much smaller response than their CD8 counterpart (**Fig 7B**), and lung-resident CD4 T cells did not respond to either VV or MPV stimulation (Fig 7B). There is a possibility that CD4 T cells might act through a different mechanism than IFNγ or TNFα secretion and we therefore were unable to detect antigen-specific CD4 T cells in using our current staining scheme. To address this possibility, we are currently investigating whether CD4+ cells are alternatively secreting IL-2 and/or IL-4.

Knowing that the MPV-infected animals have T cell responses to MPV, we have initiated studies to define the antiviral CD4+ and CD8+ T cell and antibody responses to the MPV structural proteins, A29L and A35R. T cell responses from the blood and BAL will be evaluated using overlapping peptides and antibody responses from sera will be determined by western blot analysis with whole virus and later with recombinant proteins.

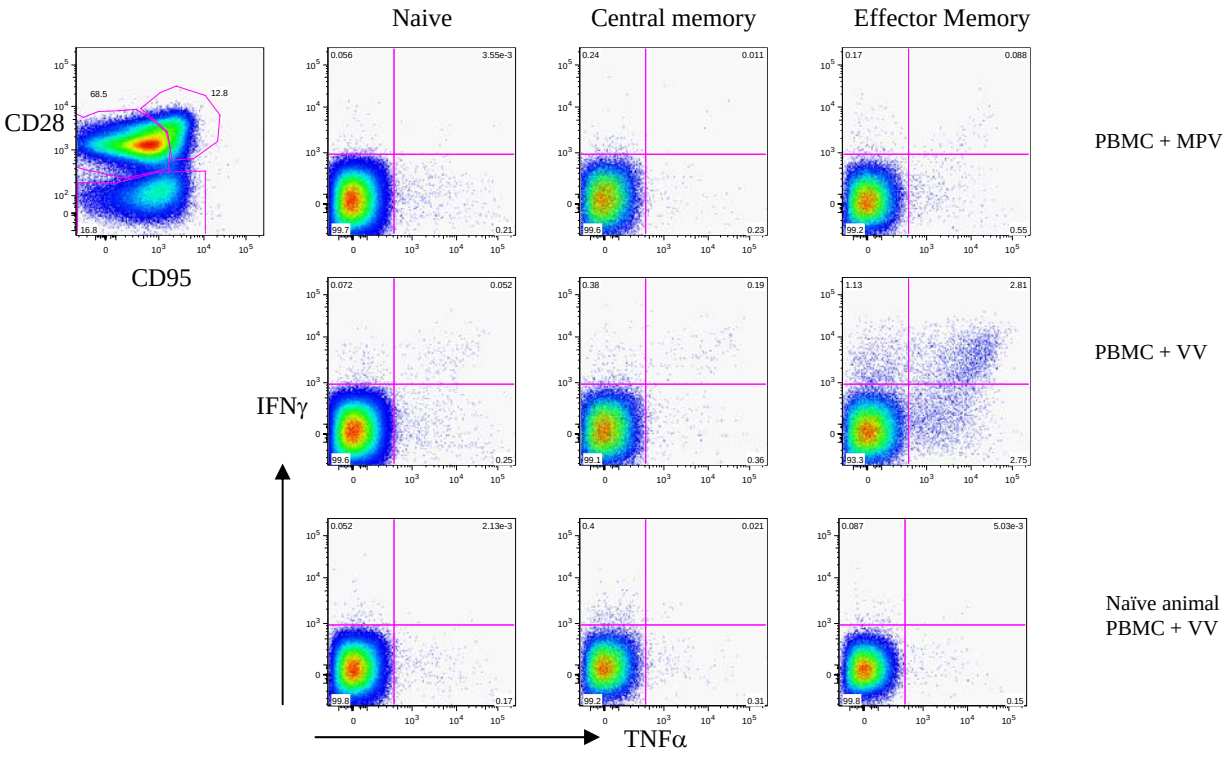


Figure 6 CD8+ T cell responses to MPV in MPV-infected or naïve animal. (A) PBMC from MPV infected or naive animals were stimulated with either MPV, VV-WR strain as described previously in Hammarlund et al. (1). Frequency of IFNγ and TNFα secreting T cells was determined using intracellular cytokine assay. Stimulation of PBMC with VV leads to a strong cytokine response dominated by CD8 T cells. Surprisingly, stimulation of PBMC with MPV did not result in a detectable cytokine response, indicative of immune evasion mechanisms employed by MPV.

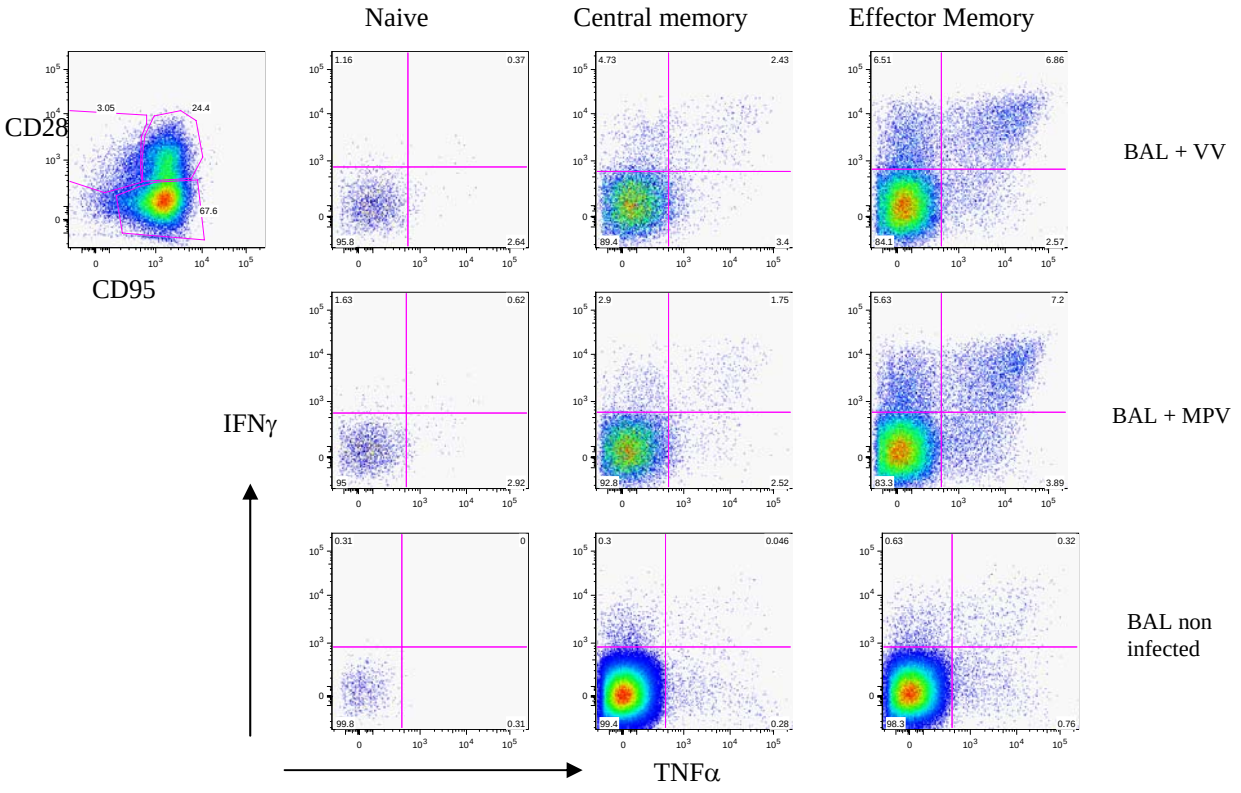


Figure 6 CD8+ T cell responses to MPV in MPV-infected or naïve animal. (B) Same analysis described in Figure 5A was carried out on T cells isolated from bronchial alveolar lavage (lung wash - BAL). Interestingly, stimulation with both VV and MPV resulted in a strong cytokine response, once again mostly mediated by CD8 T cells. MPV was not able to subvert the CD8 response in this tissue.

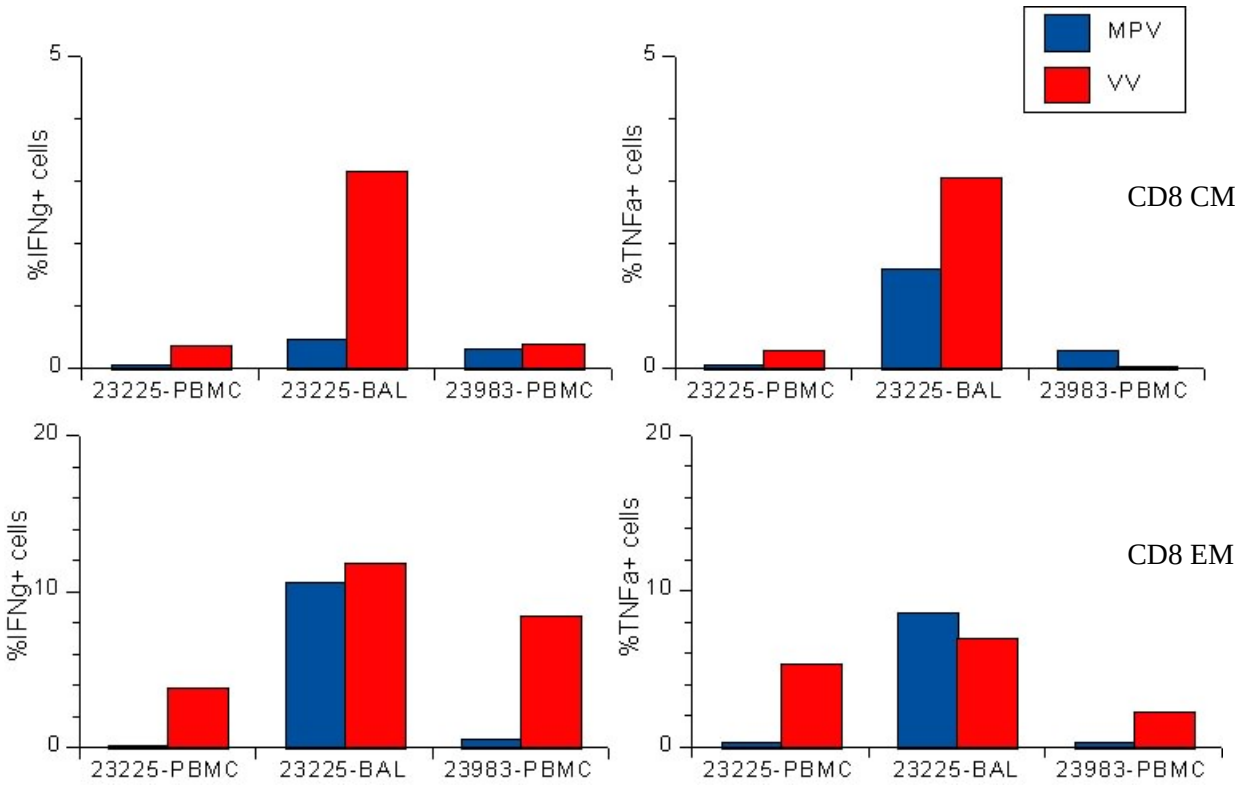


Figure 7 Graphical comparison of T cell responses from PBMC and BAL. (A) This figure shows a comparison of representative CD8 T cell responses generated in both PBMC and BAL. The data is divided amongst the two major memory subsets: central memory (CM) and effector memory (EM). A higher percentage of cells with the CD8 EM subset secrete IFNg and TNFa after stimulation with VV and MPV especially in BAL. This is expected since the lung was the site of infection.

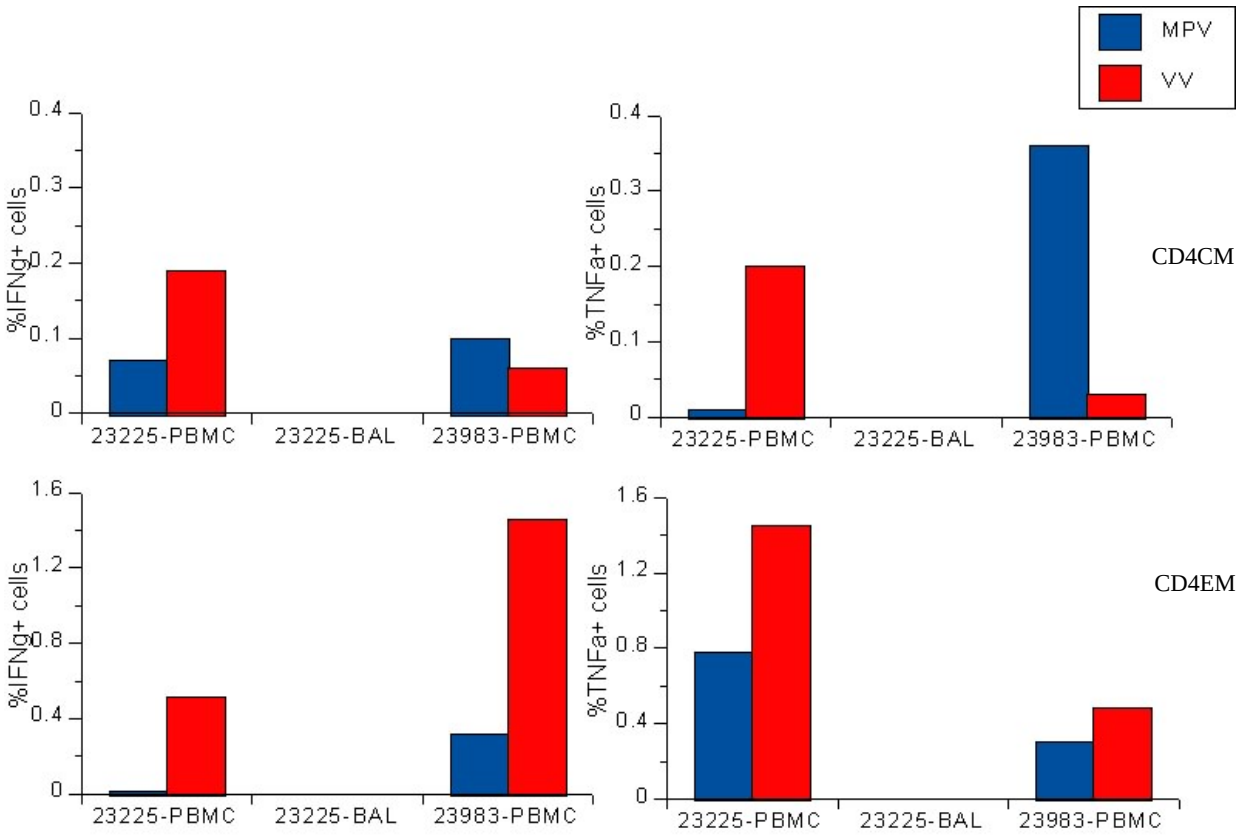


Figure 7 Graphical comparisons of T cell responses from PBMC and BAL. (B) Summary of representative CD4 responses. CD4 T cells responses were lower than CD8 T cells responses, but followed similar hierarchy with CD4 EM responding more vigorously. Interestingly we were not able to detect any CD4 T cells in BAL.

KEY RESEARCH ACCOMPLISHMENTS:

Construction and in vitro characterization of RhCMV/MPV vaccine vector
Defining pathologic outcome of intrabronchial MPV-inoculation
Defining host response to MPV infection with biotelemetry
Characterization of host immune response to pathogenic MPV infection

REPORTABLE OUTCOMES:

None

CONCLUSIONS:

We have accomplished or are nearing completion of the specific aims proposed in the initial application. In Specific Aim 1, we were successful in creating two series of RhCMV vectors each containing one of four individual MPV genes (A29L, A35R, M1R and B6R). Animals are currently scheduled to be experimentally inoculated with these vectors and the animals will be extensively characterized to assess immunological responses to the MPV antigens. Specific Aim 2 has been completed. As such, we have established the intrabronchial inoculation MPV: RM model and are well on are way to complete the studies originally described in Specific Aim 3, which is to characterize the host immune response to MPV A35R and A29L. Taken together, we are now well positioned to define the efficacy of RhCMV/MPV vectors to elicit a protective immune response to MPV challenge in the rhesus macaque animal model.

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Review of Annual Report April 2007

REVIEWER: Pamela J. Glass, Ph.D.

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TITLE: Development of a Novel Vector for Multiple CDC Category A Pathogens

PRINCIPAL INVESTIGATOR: Jay A. Nelson, Ph.D.
Scott W. Wong, Ph.D.
Michael A. Jarvis, Ph.D.

This annual report was submitted at the conclusion of year two of funding for the proposal.

The investigators report that specific aim 1 is complete resulting in the construction of two series of RhCMV vectors each containing one of four individual MPV genes (A29L, A35R, M1R and B6R). Restriction enzyme digestion and southern blot analysis of all recombinant RhCMV/MPV vectors is presented (Fig. 2). The original proposal indicates:

> "Upon identification of recombinant RhCMV BACs, the recombinant BAC DNA will be electroporated into RM fibroblasts for the production of BAC-derived recombinant virus. An amplified stock of recombinant virus will be used to test for MPV antigen production by immunofluorescence and western blot analysis using antibodies to the epitope tags incorporated into each vector. Viral fitness and tropism will be assessed *in vitro* by viral growth kinetics in RM fibroblasts, EC and MDM."

No protein expression analysis from these recombinant vectors was shown except for the WT and Δ versions containing the A35R coding region (Fig. 3). The A35R expression data was presented in the context of preliminary experiments, beyond the scope of this proposal, which will examine the immune response against the MPV target antigens in RMs. The exclusion of data or mention makes me wonder if any of the other constructs express the encoded MPV target antigen. Additionally there was no mention of viral fitness and tropism analysis being completed or in progress in either of their annual reports.

Specific aim 2 is currently ongoing and the investigators are making progress toward defining the pathobiology of WT MPV infection in nonhuman primates. In this annual report's conclusion section, the PI indicates this aim has been completed; however the aims and milestones that were expected to be achieved as stated in their original proposal have not been completed or even mentioned in their annual reports. The investigators indicated they would be determining a lethal dose by examining animals in 4 groups inoculated with different doses of MPV. To date it appears from their annual reports that 3 groups have been examined with 2 different doses of MPV. One group received 2×10^7 pfu and two groups received 2×10^5 pfu. From these experiments, they have accumulated significant clinical disease progression as well as gross and histopathological examination data for MPV infection of nonhuman primates. They have isolated virus from the fluids and other organ tissues or cells. The investigators indicate that further evaluation of viral load is being performed by real-time PCR in both annual reports. Yet in their original proposal, the RT-PCR assay development and correlation with virus titer was to be determined to allow correlation of these methods with disease progression. The original proposal also mentioned that the role of the macrophage in *in vivo* spread would be examined. There is no mention of the progress of these experiments or analysis in either annual report.

Specific aim 3 also is currently ongoing and the investigators appear to be making progress toward determining the host immune response to WT MPV infection in nonhuman primates. However, the results presented in this section of the annual report were difficult to follow and clearly determine the outcome of the experiments. In Fig. 7, the data presented is designated by animal numbers; however, no animal numbers were presented within the text or figure legend to indicate the "treatment" each animal received.

While significant progress and important data has been generated from these experiments, the investigators indicate that specific aims 1 and 2 are done yet many of the milestones from the original proposal have not been completed or addressed in their annual reports.

Dear Dr. Glass,

We have addressed you concerns with the results from our studies in a point-by-point fashion below. Please note that we requested a no-cost extension to complete our studies in March 2007. A final report will be submitted in 2008.

1. "No protein expression from the recombinant vectors was shown except for the WT and deleted versions containing the A35R region (Figure 3)…The exclusion of data or mention makes me wonder if any of the other constructs express the encoded MPV antigen target antigen."

Response: As indicated in the report, we decided to move beyond the initial scope of the study by assessing immunogenicity in vivo. We believed that immunogenicity studies in rhesus primates was far more of a scientifically and clinically important endpoint than one of a panel of in vitro characterized recombinants. We therefore decided to use the crude reconstituted virus lysate as an ideal 'stopping point', since the ability to reconstitute virus would confirm, albeit non-quantitatively, the ability of recombinant viruses to replicate in vitro. The A35R WT and deleted recombinant were arbitrarily chosen for the in vivo studies, and these viruses were serially passed (to remove the BAC cassette), expanded into stocks, and then confirmed for A35R protein expression. We subsequently decided to focus on the WT version since results from an ongoing NIH-funded study indicated that RhCMV viruses deleted for immunomodulatory genes cannot establish infection in RhCMV seropositive animals (Dr. K. Frueh, privileged information). As you indicate, there is always the possibility that target protein expression will be inadvertently lost in the recombinant reconstituted virus, for example due to selective pressure against overexpression of a toxic protein. However, genomic characterization of all viruses (except of course the B6R WT virus) indicated genomic integrity of all recombinants at the BAC DNA level (Figure 2A and 2B), and we therefore expect that all reconstituted viruses will express the respective MPV target protein, as was observed with A35R recombinants.

2. *"Additionally, there was no mention of viral fitness and tropism analysis being completed or in progress in either of their annual reports".*

Response: We believe that the in vivo studies are the definitive test for viral fitness. Our experience with multiple RhCMV vectors expressing SIV antigens reveal no growth defects in recombinants expressing heterologous antigens. Since the A35R WT recombinant was observed to grow to normal titers during production of the virus stock, in vitro growth analysis of this virus was not a priority. We do however plan to confirm WT in vitro growth kinetics prior to publication of results from the in vivo studies.

3. *"In this annual report's conclusion section, the PI indicates this aim has been completed; however the aims and milestones that were expected to be achieved as stated in their original proposal have not been completed or even mentioned in their annual reports. The investigators indicated they would be determining a lethal dose by examining animals in 4 groups inoculated with different doses of MPV. To date it*

appears from their annual reports that 3 groups have been examined with 2 different doses of MPV. One group received 2 x 10^7 pfu and two groups received 2 x 10^5 pfu."

Response: Our initial experimental animal infection with 2 x 10^7 PFU was a lethal dose. We subsequently decreased the inoculum to determine if 2 x 10^5 PFU was capable of inducing lethal disease in a second group of animals (Group 2; 23225 and 23983). At this dose, animal 23983 developed pox lesions by day 4 post-infection (pi) and was euthanized on day 17 post-infection due to respiratory disease. Animal 23225 also developed pox lesions on day 4 pi, but was euthanized at day 111 post-infection, well after resolution of acute disease. Samples were collected from this animal and analyzed immunologically to define host response to MPV and were presented in original Figure 6.

Another group of animals (Group 3; 23218 and 23358) were then inoculated with 2 x 10^5 PFU to further investigate this dose. As written in our annual review, both animals survived implying that 2 x 10^5 PFU is in fact a sub-lethal dose when delivered intrabronchially. Animal 23358 developed pox lesions on day 4 pi, while animal 23218 developed systemic disease on day 7pi. These animals were further evaluated immunologically and euthanized at day 39 pi.

Since 2 x 10^5 PFU is not a lethal dose, we intend to inoculate two animals (Group 4) with 2 x 10^6 PFU to experimentally determine whether this dose is lethal. Based upon our experimental infections studies performed to date, we anticipate this dose will be lethal.

4. *"The investigators indicate that further evaluation of viral load is being performed by real-time PCR in both annual reports. Yet in their original proposal, the RT-PCR assay development and correlation with virus titer was to be determined to allow correlation of these methods with disease progression. The original proposal also mentioned that the role of the macrophage in in vivo spread would be examined. There is no mention of the progress of these experiments or analysis in either annual report."*

Response: We are currently in the process of optimizing the MPV real-time PCR assay to improve sensitivity. We intend to have these samples analyzed once the assay is well established in our hands.

5. *"The original proposal also mentioned that the role of the macrophage in in vivo spread would be examined. There is no mention of the progress of these experiments or analysis in either annual report."*

Response: Dr. Glass is correct. We have verified that the animals develop a monocytosis and we intend to define the viral load in these samples to correlate this with spread of disease. Our initial results strongly suggest that monocytosis correlates with spread of disease, as appearance of lesions on the extremities coincides with onset of monocytosis.

6. *"However, the results presented in this section of the annual report were difficult to follow and clearly determine the outcome of the experiments. In Fig. 7, the data presented is designated by animal numbers; however, no animal numbers were presented within the text or figure legend to indicate the "treatment" each animal received."*

Response: Dr. Glass is correct. Below is a modified discussion to better explain our immunological data accumulated to date for Specific Aim 3: *We will monitor the immunological consequences of WT MPV infection of non-human primates*.

To define the host immune response to MPV infection, we analyzed antigen specific T cell responses following MPV challenge as described by Hammarlund et al., (2005). Briefly, peripheral blood mono-nuclear cells (PBMC) or bronchial alveolar lavage (BAL) cells from either infected (Group 2 animal 23225) or naïve animals were stimulated with either vaccinia virus (VV) or monkeypox virus (MPV) at an MOI of 1. After a 12 hr incubation, brefeldin A was added to block cytokine export from the endoplasmic reticulum /Golgi for an additional 6hr. Cells were then washed and stained with anti-CD8, CD4, CD95 and CD28 to delineate T cell subsets. Both CD8 and CD4 T cells can be further subdivided into three subsets based on the pattern of expression of CD28 and CD95: 1) naïve (CD28+CD95-); 2) central memory (CD28+CD95+); and 3) effector memory (CD28-CD95+). After the surface staining, the cells were permeabilized and antibodies against IFNγ and TNF-α were added. All samples were acquired on the LSRII using the FACsDIVA software (Becton Dickenson, San Jose, CA) and analyzed using FlowJo (Treestar, Ashland, OR).

Our data shows that following MPV infected animal (23225) generated a strong T cell response dominated by the effector memory (EM) CD8 subset (Fig 5A). Interestingly, EM CD8 T cells present in PBMC secrete higher levels of IFNγ in response to vaccinia stimulation than MPV stimulation. This might be indicative of immune evasion mechanisms by MPV that dampen the adaptive immune response in the periphery. This difference, however was not observed in the CD8 T cells obtained from the BAL (Fig 5B) where the IFNγ responses generated after MPV or VV infection were equivalent (Fig 6 A). This discrepancy could be due to the different antigen presenting cell populations present in the lung versus peripheral blood. Alternatively this difference could be due to the difference in activation status of the T cells recruited to the site of infection (lung) versus those that circulate in peripheral blood. We are currently investigating these different possibilities by studying the levels of both MHC class I and II molecules following VV and MPV infection in PBMC and BAL cells. We are also analyzing the resident cell populations in the lung that could act as antigen-presenting cells using multicolor flow cytometry.

Interestingly, we observed that peripheral CD4 T cells generated a much smaller response than their CD8 counterpart (Fig 6B), and lung-resident CD4 T cells did not respond to either VV or MPV stimulation (Fig 6B). This could suggest that CD4 T cells might act through a different mechanism than IFNγ or TNFα secretion and we therefore were unable to detect antigen-specific CD4 T cells in using our current staining scheme. To further define whether this was unique to Group 2 animals (23225 and 23983), we performed similar analyzes on samples collected from Group 3 animals (23218 and 23358) who were infected intrabronchially with $2x10^5$ PFU MPV Zaire strain. As mentioned above, Group 3 animals were euthanized at day 32 pi and several tissues were analyzed for the presence of MPV-specific CD8 and CD4 T cells by ICCS analysis (Fig 7). The data presented shows the % IFNγ (IFNg) secreting CD4 and CD8 T cells detected in PBMC or BAL following stimulation with either VV or MPV at an MOI of 1. We also

measured the frequency of TNFα secreting T cells, however the percentage of TNFα + CD4 and CD8 T cells was negligible compared to the IFNγ+ T cells (data not shown). By this analysis, we found the overwhelming majority of the responding T cells are CD8+ and the frequency of MPV specific CD8 T cells was higher in BAL than PBMC. Furthermore, in BAL, a higher frequency of CD8 T cells responded to MPV stimulation than VV stimulation as seen in samples from our earlier cohort. These data indicate that CD8 T cells in the BAL are responding to viral antigens uniquely expressed by MPV (not present in VV).

Knowing that the MPV-infected animals have T cell responses to MPV, we have initiated studies to define the antiviral CD4+ and CD8+ T cell and antibody responses to the MPV structural proteins, A29L and A35R. T cell responses from the blood and BAL will be evaluated using overlapping peptides and antibody responses from sera will be determined by western blot analysis with whole virus and later with recombinant proteins.

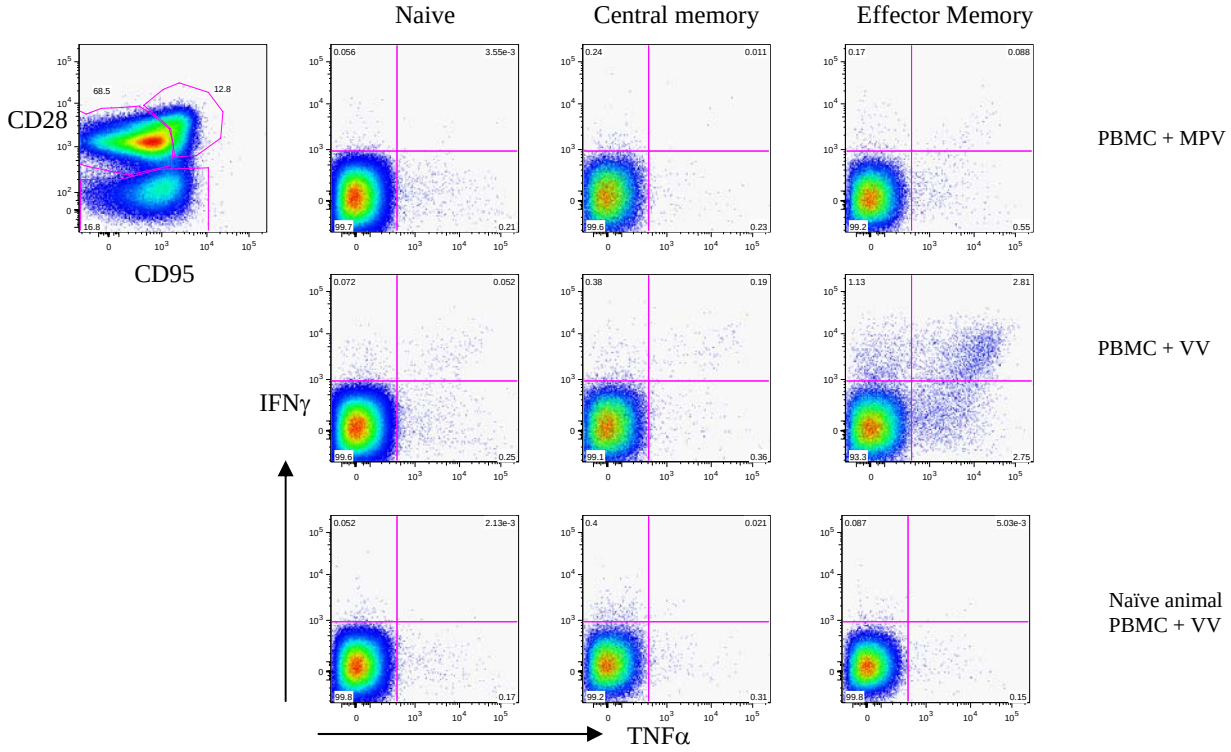

Figure 5A CD8+ T cell responses in the peripheral blood to MPV in MPV-infected or naïve animal. PBMC from MPV infected or naive animals were stimulated with either MPV, VV-WR strain as described previously in Hammarlund et al. (2005). Frequency of IFNγ and TNFα secreting T cells was determined using intracellular cytokine assay. Stimulation of PBMC with VV leads to a strong cytokine response dominated by CD8 T cells. Surprisingly, stimulation of PBMC with MPV did not result in a detectable cytokine response, indicative of immune evasion mechanisms employed by MPV.

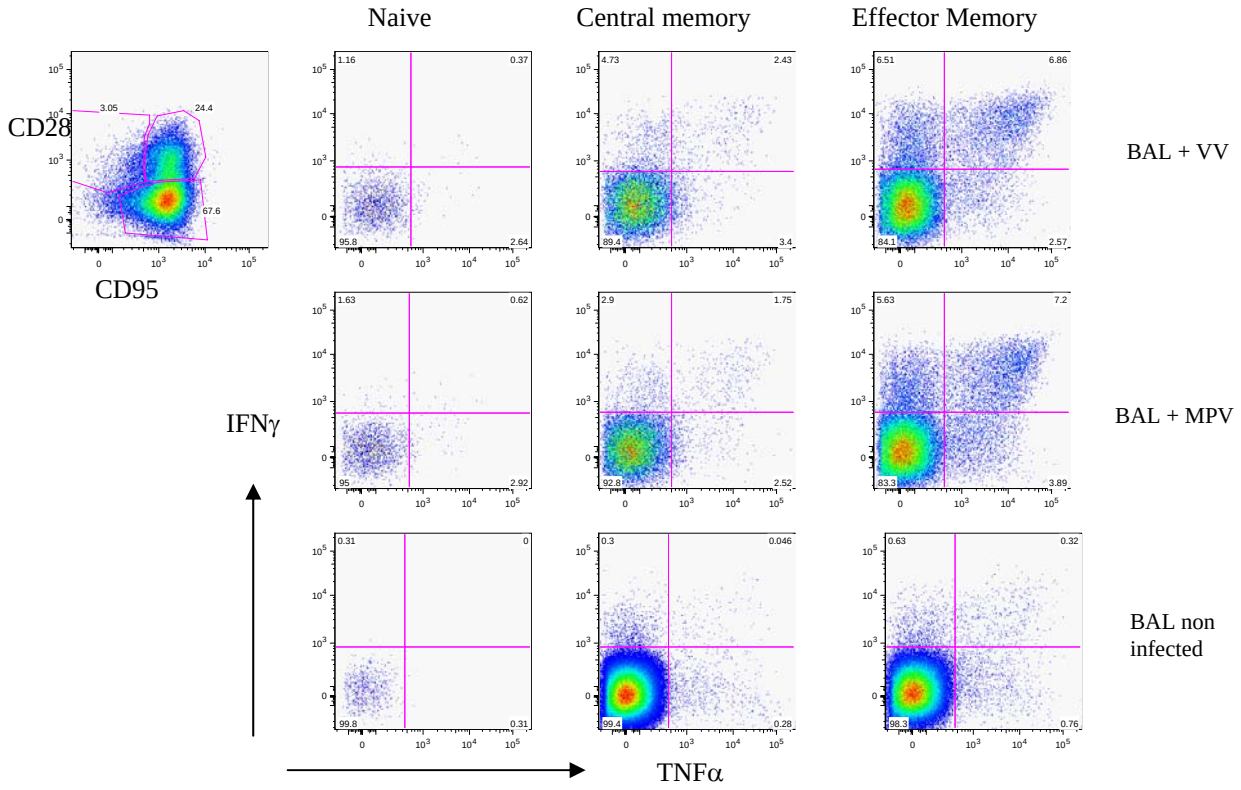

Figure 5B CD8+ T cell responses in bronchial alveolar lavage to MPV in MPV-infected or naïve animal. Same analysis described in Figure 5A was carried out on T cells isolated from bronchial alveolar lavage (lung wash -BAL). Interestingly, stimulation with both VV and MPV resulted in a strong cytokine response, once again mostly mediated by CD8 T cells. MPV was not able to subvert the CD8 response in this tissue.

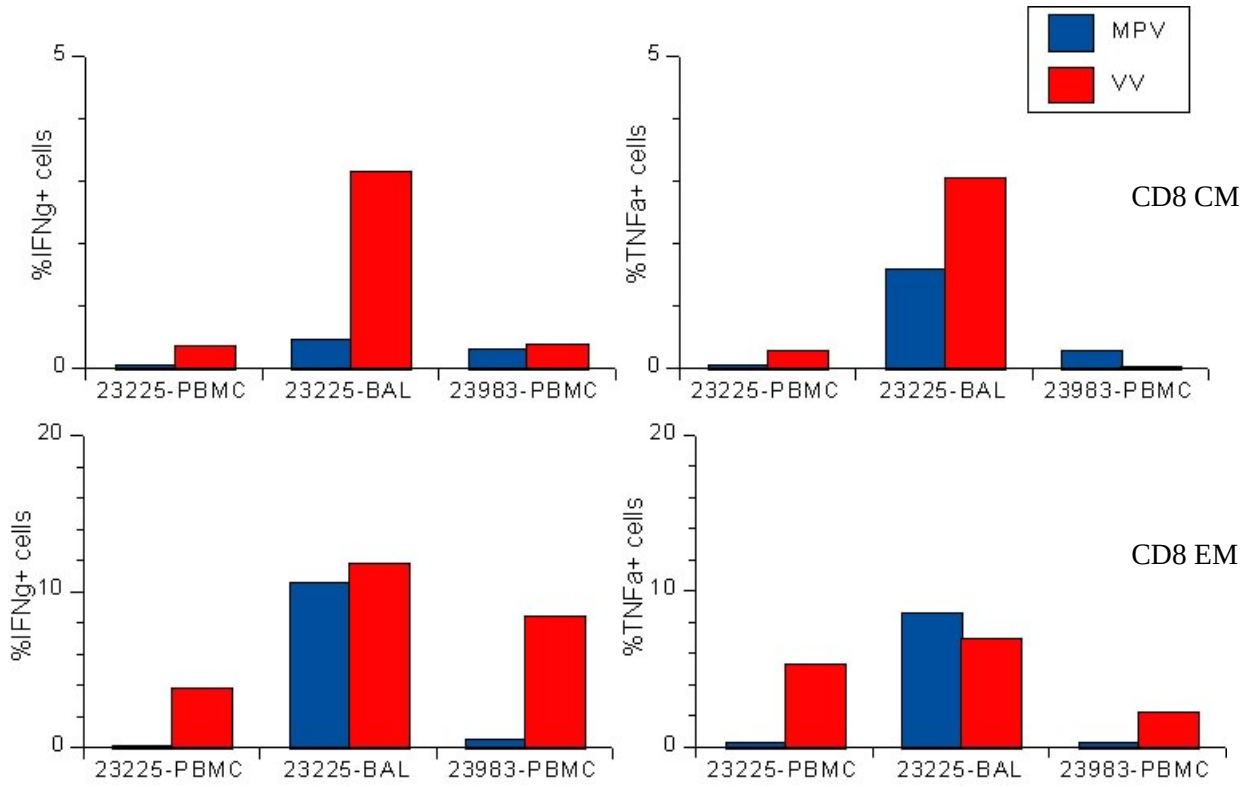

Figure 6A This figure shows a comparison of representative CD8 T cell responses generated in both PBMC and BAL. The data is divided amongst the two major memory subsets: central memory (CM) and effector memory (EM). A higher percentage of cells with the CD8 EM subset secrete IFNg and TNFa after stimulation with VV and MPV especially in BAL. This is expected since the lung was the site of infection. We were unable to collect BAL from animal 23983, so this sample was not analyzed for CD4 and CD8 T cell responses.

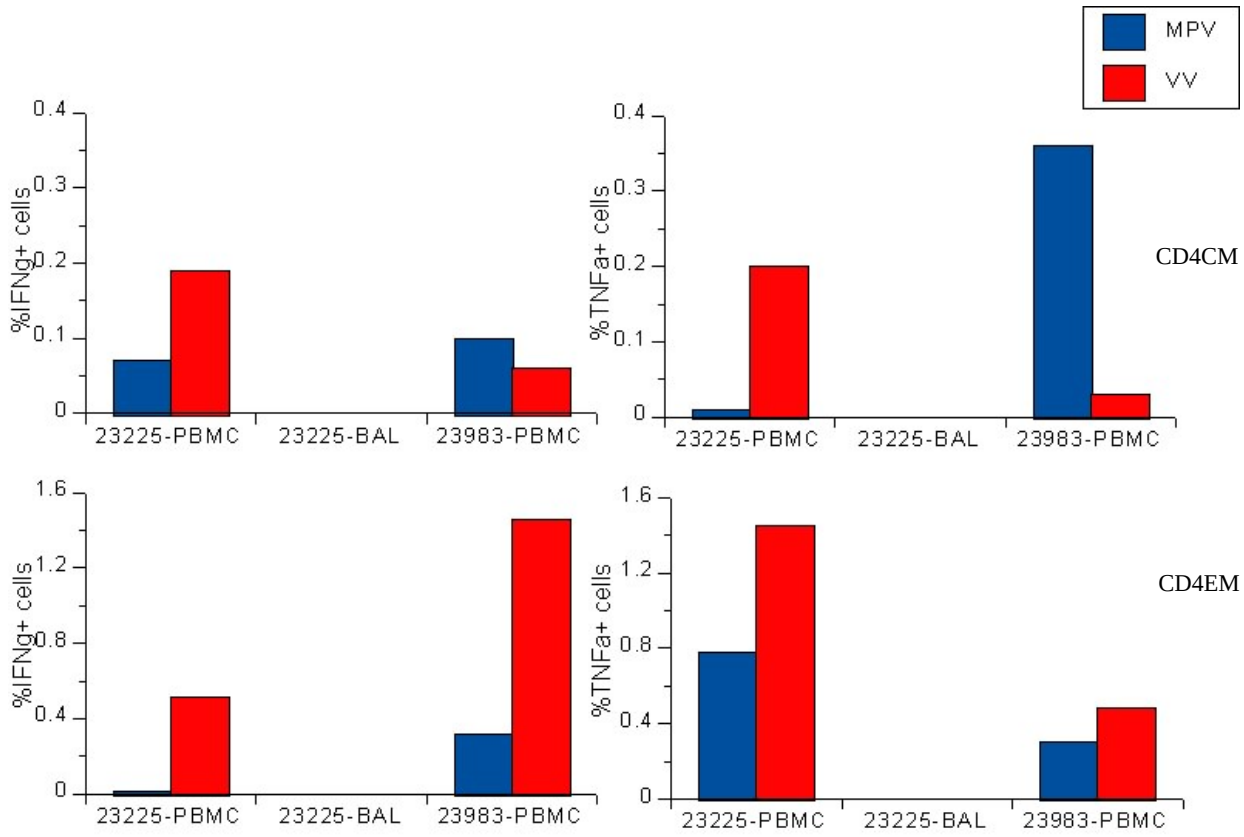

Figure 6B Summary of representative CD4 responses. CD4 T cells responses were lower than CD8 T cells responses, but followed similar hierarchy with CD4 EM responding more vigorously. Interestingly we were not able to detect any CD4 T cells in BAL.

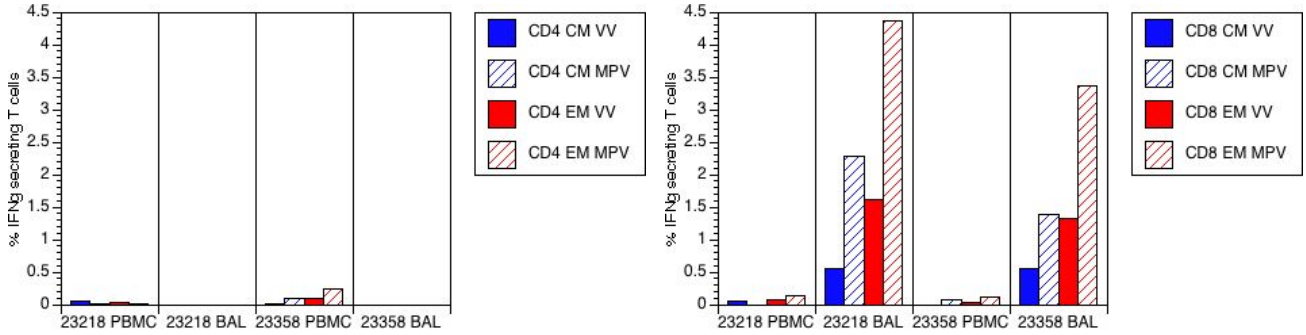

Figure 7 Summary of CD4 and CD8 responses in Group 3 animals (23218 and 23358). CD4 T cell responses were almost not detectable, whereas CD8 T cell (IFNg) were much more detectable in the BAL versus the peripheral blood.